The impact of random natural variability on aspartic acid racemization ratios in enamel from different types of human teeth

Abstract

Previous research has indicated that the extent of amino acid racemization in enamel varies systematically between tooth types within the dentition. This phenomenon was suggested to be due to differences in temperature at various locations within the mouth. This paper presents an analysis of aspartic acid racemization in a fraction of the enamel proteins which should be particularly susceptible to deviations in temperature, in order to assess the impact of temperature on variability in racemization values. The acid soluble fraction of the enamel was analysed from 129 human teeth of different tooth types and from both living individuals and archaeological skeletal remains. Samples were collected by acid etching of the enamel to isolate proteins located a small distance below the enamel surface. For each population, the racemization values for different tooth types were compared to identify any possible systematic variation. Where multiple teeth were analysed from the one individual, the age estimates produced for the different teeth were compared to obtain an indication of the overall level of variability in racemization values. No systematic variation in the extent of racemization between different tooth types was observed in any of the populations analysed. There appeared instead to be a high level of random variability in the extent of racemization, with substantial differences observed between age estimates produced from multiple teeth from the one individual. The results of this study suggest that the differences in racemization values observed here are due to random variations and not the temperature at different locations within the mouth.

Keywords

aspartic acid racemization, enamel, age at death estimation, tooth type, variability, intra-individual differences

Introduction

One of the key challenges facing forensic anthropology is the estimation of age at death of skeletonised remains. For juvenile remains, dental development is generally considered to be one of the most reliable methods for estimating age at death [1]. However, for adult remains the accurate estimation of age at death remains a problem. While a plethora of age estimation methods have been developed, the majority have been found to be fairly inaccurate, and to produce as a result only very broad age estimates [e.g. 1, 2, 3]. This is due to the emphasis of most age estimation techniques on degenerative changes that occur as part of the aging process, which are highly susceptible to variations between individuals in the timing of their occurrence. One possible solution to this problem is to develop age estimation methods which utilise chemical processes that occur with age, as the rate of occurrence of these processes should not be as susceptible to variations between individuals. Amino acid racemization is one such chemical age estimation technique which has been adopted successfully within forensic research [e.g. 4, 5-7].

Amino acid racemization is a change which occurs in amino acids during life. Most amino acids within the human body can exist as either an L-isomer or a D-isomer, which are non-superimposable mirror images of each other. All proteins in the human body are initially formed with amino acids in the L- form, but as this situation is thermodynamically unstable, in proteins that are not turned over by the body the amino acids can undergo racemization *in vivo* to ultimately form an equilibrium mixture of L- and D- amino acids. The racemization reaction is strongly temperature dependent, so in temperate environments racemization slows significantly post-mortem if an individual is buried shortly after death. As a result, little racemization should take place over short post-mortem intervals of up to a few decades, allowing determination of age at death from the relative proportions of L- and D-amino acids in proteins which were formed early in development and which have not undergone biochemical turnover during life. While the rate of racemization post-mortem could potentially be affected by soil content, pH and hydrology, the isolation of intra-crystalline amino acids, which are believed to be act as a closed system and therefore less susceptible to these factors, can minimise the effect of these variables on the rate of racemization post-mortem [8]. Although amino acid racemization age estimation can utilize a range of different tissue types, it is most commonly applied to dental tissues, particularly dentine and enamel [9, 10], due to their formation early in the life of an individual.

Given the different ages at which the various tooth types form, the D/L values obtained from multiple teeth from the same individual would be expected to vary depending on the age of formation of each tooth. However, studies of multiple teeth from individuals of known age by Ohtani et al. [11, 12] has indicated that differences in racemization between tooth types exist in both dentine and enamel, which cannot solely be explained by the age at formation of each tooth type. If this is so, this may impact upon tooth selection for age at death estimates. Some of the observed variation appeared to be linked to the location of each tooth within the mouth. Ohtani et al. [11, 12] therefore hypothesized that these variations were due to differences in the temperature experienced by teeth, depending upon their location within the jaw.

This paper seeks to investigate whether differences in the extent of aspartic acid racemization in the acid soluble fraction of enamel in teeth from diverse tooth types are related to differences in their location within the mouth rather than variations in their age at formation. As enamel has been suggested to be better preserved over longer periods of burial than dentine, it may be of use in forensic age estimation for remains with a longer post-mortem interval without encountering the problems observed when applying AAR to dentine from historical remains [5]. The fraction to be used here is the acid soluble portion of the enamel, in which there is a close relationship between amino acid racemization and age [13]. The proteins sampled using the method of Griffin et al. [13] are located close to the surface of the enamel, and thus should be more susceptible to any changes in temperature experienced by the tooth as a whole than the total enamel protein content. This paper therefore aims to determine whether there is a systematic variation by tooth type in the extent of racemization in the acid soluble fraction of enamel, and what the causes are of any variations observed in the extent of racemization between teeth.

Materials and methods

Materials

The data included in this study were from teeth analysed as part of two broader studies which attempted to use amino acid racemization in the acid soluble portion of the enamel for age estimation purposes [13, 14]. The acid soluble fraction was selected for analysis, as this fraction can be collected using minimally-destructive sampling, which is not possible when analyzing the total enamel content. The teeth in this study include modern teeth extracted from individuals of known age, teeth from known age at death individuals from the 19th century cemetery of

Spitalfriedhof St Johann in Basel, and teeth from individuals without documented ages at death from the medieval cemetery of Grantham and the early medieval cemetery of Newcastle Blackgate. By examining racemization data from a range of populations, it should be possible to determine whether any systematic variations observed are population specific, or are potentially more universally applicable.

The modern sample included in this study was comprised of thirty one healthy teeth (2 I_1, 1 I_2, 1 C^1, 2 C_1, 2 P_1, 1 M^1, 1 M_1, 9 M^3, 9 M_3 and 3 dm_2) with known age of extraction, provided by the Edinburgh Dental Institute and the Clockhouse Dental Surgery, York. The teeth were selected from those donated to cover the broadest age range possible. After extraction, the teeth were rinsed with bleach and sent to the laboratory in York either dry (for teeth from the Edinburgh Dental Institute) or in bleach (for teeth from the Clockhouse Dental Surgery). These teeth were used to calibrate the method, in accordance with the recommendations of Ritz-Timme et al. [15].

In addition to the modern extracted teeth, a number of healthy teeth were analysed from three archaeological populations. These included 13 teeth from the early medieval (8^{th}-10^{th} century AD) site of Newcastle Blackgate (1 C_1, 1 P^1, 1 P_2, 1 M^1, 1 M_1, 2 M^2, 4 M_2, 1 dm_1 and 1 dm_2), 14 from the medieval cemetery of Grantham (c. 15^{th} century AD; 2 P_2, 1 M_1, 3 M_2, 4 M_3, 2 dm_1, 2 dm_2), and 71 from the Spitalfriedhof St Johann known age reference collection from Basel (1845-1868 AD; 1 C^1, 2 C_1, 3 P^1, 5 P^2, 7 P_2, 6 M^1, 9 M_1, 5 M^2, 19 M_2, 6 M^3, 8 M_3). All the teeth from Newcastle Blackgate and five of those from Grantham were from commingled remains, which could not therefore be connected with particular individuals, while those from Spitalfriedhof St Johann and the remainder of those from Grantham belonged to individual skeletons. The teeth from Newcastle Blackgate and Grantham were selected to include only those which could be removed from the jaw for analysis. The teeth from Spitalfriedhof St Johann were selected by Dr Gerhard Hotz to cover as wide a range of adult ages at death as possible.

Methods

The labial and lingual surfaces of each tooth were cleaned by applying a 0.2 mL PCR tube filled with 6 M HCl (total volume approximately 230 μL) to each surface for one minute, then rinsing the surfaces with HPLC grade methanol. In order to remove any contaminant proteins from the tooth surface the teeth were placed in 12% wt/vol sodium

hypochlorite for two days, and then rinsed in ultrapure water and HPLC grade methanol. The labial and lingual surfaces of each tooth were then sampled by applying two 0.2 mL PCR tubes filled with 6 M HCl for two consecutive time intervals of one minute. The teeth from Newcastle Blackgate were sampled for another two consecutive time intervals of one minute, as these teeth were also used in the development of the sampling methodology. Therefore the portion of the enamel sampled was deeper for the teeth from Newcastle Blackgate than for the other sites studied. While the depth of sampling has been shown to have an impact upon Asx D/L [16], after a certain depth of sampling and with treatment of the enamel with bleach prior to sampling, the Asx D/L values plateau. All the samples examined in this study were taken from fractions within this plateau, including those from Newcastle Blackgate. This was confirmed by the presence of low Glx D/L values in these samples, found in Griffin et al. [16] to be associated with the fractions within the plateau region. Therefore, we would expect the deeper sampling of the teeth from Newcastle Blackgate to have a minimal effect upon the Asx D/L values obtained. Furthermore, the amino acid profiles of these samples were not significantly different from those obtained for the other archaeological sites, suggesting that the protein fraction analysed from all the populations was the same (the typical amino acid compositions of the samples can be found in Griffin et al. [13, 14]).

The second sample from each tooth surface was hydrolysed at 110 °C for six hours in an N_2 enriched atmosphere in sterile glass vials. The samples were then dried overnight on a centrifugal evaporator. When dry, the samples were rehydrated in 50 μL of 0.003 M HCl, 0.01 mM L-homo-arginine (as an internal standard) and 0.77 mM sodium azide, with a pH of 2. The sample vials were vortexed to aid dissolution, centrifuged and the supernatant collected and analysed on the HPLC. Samples were separated on a Hypersil BDS C_{18} column by reverse phase HPLC using a modified version of the method of Kaufman and Manley [17]. Three analyses were conducted for each sample, one using a shortened version of the chromatographic method of Penkman et al. [8], which allowed the separation of L-Asx (L-aspartic acid and L-asparagine), D-Asx, L-Glx (L-glutamic acid and L-glutamine), D-Glx, L-Ser (L-serine), D-Ser, Gly (glycine), L-Ala (L-alanine) and L-homo-arginine (Figure 1), and the second and third using a variation on the previous method with the concentration gradients halved in order to improve the separation of the L- and D-Asx peaks. Absolute concentrations of L- and D- amino acids were determined from the integrated area under each peak, normalized to the peak area of the internal standard, L-homo-arginine. The average value of Asx D/L from

the second and third analyses of each sample was then determined. For quality control purposes, amino acid standards and blanks were also analysed daily, as recommended by Ritz-Timme et al. [15].

For a number of individuals, it was possible to analyse multiple teeth from the one individual. Of these, three were living individuals who had donated multiple teeth, seven were from Newcastle Blackgate, four were from Grantham and six were from Spitalfriedhof St Johann. In most cases, two teeth were analysed from each individual; however, three teeth were analysed from one living individual and one individual from Newcastle Blackgate, and four teeth were analysed from each of two individuals from Newcastle Blackgate. By comparing the results obtained from multiple teeth from a single individual it should be possible to estimate the level of variability in the extent of racemization in the teeth of a given individual.

Statistical methodology

For each tooth, the D/L values obtained from the two sides of the tooth sampled were averaged, and this value was converted into an age estimate by applying a log transformation, and then using the regression equation of Griffin et al. [13]. Errors associated with the application of this regression analysis are presented in Griffin et al.[13]. Because a range of different tooth types were included in this study, the tooth ages were corrected for the different ages of formation of each tooth type after applying the regression equation. When estimating age from Asx D/L, this was done by adding to the estimated age the average age at crown completion for each tooth reported in Hillson [18]. Where the sex of the individual was known, sex specific ages at formation were used. While it is possible that tooth development may not have followed the same trajectory in archaeological populations as it does in modern populations, modern timings of tooth development have been shown to produce accurate age estimates for subadults in recent archaeological samples [19]. The relatively recent date of the majority of the remains considered here will therefore minimize the impact of any differences. Furthermore, by using crown formation times from populations with a similar background to those included in this study (i.e. western European and North American) difficulties due to variations in the timing of dental development between populations [e.g. 20, 21] will be minimized.

As the racemization values obtained from the archaeological populations will also contain a component of racemization resulting from the post-mortem period, it was necessary to correct the age estimates for these

individuals to take post-mortem racemization into account. Age estimates for the Newcastle Blackgate and Grantham populations were corrected for racemization occurring in the post-mortem interval by calculating for all juvenile individuals the average difference between the age estimate provided by racemization and the age estimate produced from dental development, and subtracting this amount from all the age estimates produced [13]. Juveniles were used for this purpose, as morphological age estimates for juveniles are relatively accurate [22], allowing the extent of racemization post-mortem to be determined for each juvenile. For the Basel population, juveniles were not available for analysis, so the nine youngest individuals were used to correct the age estimates instead. By determining the approximate extent of racemization taking place post-mortem at each site in this way, it is possible to take into account differences between sites in temperature and soil conditions as well as time since burial. In practice, however, time since burial is the main determinant of the extent of racemization post-mortem [23].

Results

Comparison of the results obtained for the individuals from each population by tooth type indicates that there is no relationship between the extent of racemization and tooth type, as long as the age of formation of each tooth type is taken into account when producing the age estimates (Figures 2, 3, 4, 5). There therefore appears to be no difference in the rate of racemization between different tooth types. This can be seen in the three populations where a strong relationship between Asx D/L and age had previously been observed (modern, Newcastle Blackgate and Grantham), where general linear modelling using SPSS 15.0 indicated that all the different tooth types analysed showed the same relationship between tooth age and Asx D/L ($R^2 > 0.86$).

Figures 2, 3, 4 and 5.

Comparison of the age estimates produced from multiple teeth from one individual shows that even between different teeth from the same individual there is a high level of variability in the Asx D/L values measured (Table 1, Figure 6). This variability does not appear to be related to the tooth type or to the age of the individual.

Figure 6

Table 1

Discussion

There seems to be little evidence for systematic variations in the extent of racemization in the acid soluble fraction of enamel from different tooth types, once differences in the age at formation of the various teeth are taken into account. The choice of tooth type does not appear to significantly affect the racemization result obtained, while the variation between teeth from the one individual does not seem to be related to the tooth type in any predictable way.

The data presented here instead indicate that there is a high level of seemingly random variability in the racemization values obtained from the acid soluble fraction of enamel. These resulted in substantial differences in the age estimates produced from different teeth from the one individual, with deviations in age estimates of up to 25 years for one medieval individual. However, there was no consistent increase with increased post-mortem interval in the differences between multiple age estimates from a single individual. The average variation between paired age estimates was 7.1 years, with a standard deviation of 5.98 years. This variation is smaller than the 95% confidence limits reported for the aspartic acid racemization methodology applied here [14], suggesting that the variations observed between different teeth from the one individual may largely reflect the level of error involved in the age estimation technique. This variability in the extent of racemization between different teeth from the same individual may also be due in part to the heterogeneity of enamel protein [24], and the variable peptide and protein content of the enamel, particularly as the amount of enamel removed by etching varies between teeth [25]. The variability observed seems to be greater overall in the archaeological samples than in the modern individuals, suggesting that changes during the post-mortem period or variations in the burial environment may be enhancing the level of variability. This may be due to the failure of the acid soluble fraction of the enamel proteins to act as a perfectly closed system in the burial environment [13].

This study failed to show a systematic variation in racemization values between different tooth types in the acid soluble fraction of the enamel similar to that observed by Ohtani et al. [11] for the total protein fraction. To some extent, this will be due to the correction of the Asx D/L values for differences in the age at formation of varying

tooth types, which was not undertaken in the Ohtani et al. study. Even taking this into account, however, the results of Ohtani et al. [11] show patterns in the variation of racemization values within the total protein fraction that were not observed in the acid soluble fraction. It is possible that the acid soluble fraction of the enamel is more susceptible to variation between teeth than the total enamel protein content. The use of different analytical techniques to quantify Asx D/L in the two studies may also have resulted in different amounts of analytical error, with the present study using HPLC and the study of Ohtani et al. [11] utilizing gas chromatography. However, the correlation between [ln((1+D/L)/(1-D/L)] and age for HPLC analysis of the acid soluble fraction of the enamel (R^2= 0.92) [14] is higher than that of the total enamel protein content analysed using gas chromatography (R^2= 0.86) [9]. The greater accuracy of the methodology used in the present study should therefore make systematic differences in Asx D/L between tooth types easier to detect.

The absence of any systematic trend in Asx D/L between tooth types after correction for age at formation is inconsistent with the hypothesis of Ohtani et al. [11] that there are variations in the extent of racemization due to the location of teeth in the jaw, as a result of temperature differences within the mouth. As the proteins examined in this study are located close to the surface of the tooth, it seems likely that these proteins would be more strongly affected by any differences in temperature between teeth than the total protein fraction. Thus, if temperature within the mouth was a factor, we would expect this fraction of the enamel proteins to show the pattern observed by Ohtani et al. [11], potentially more strongly than has been observed in the total enamel protein content . The failure of the results obtained here from the acid soluble fraction of the enamel to detect systematic variation in racemization between tooth types indicates that differences in temperature within the mouth are unlikely to explain any variation in racemization between different tooth types.

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Figure captions

Figure 1. Typical chromatogram obtained using the shortened chromatographic method.

Figure 2. ln ((1 + Asx D/L)/(1 - Asx D/L)) vs actual tooth age by tooth type for the modern extracted teeth analysed. Error bars represent 1st standard deviation about the mean for duplicate measurements from the same tooth. m2= deciduous second molar, I1= central incisor, I2= lateral incisor, P1= first premolar, M1= first molar, M3= third molar.

Figure 3. ln ((1 + Asx D/L)/(1 - Asx D/L)) vs estimated tooth age by tooth type for the Newcastle Blackgate teeth analysed. Ages for the Newcastle Blackgate population are those determined from dental development and dental wear. Y error bars represent 1st standard deviation about the mean for duplicate measurements from the same tooth. X error bars represent the errors in the morphological age estimates. m1= deciduous first molar, m2= deciduous second molar, C=canine, P1= first premolar, P2=second premolar, M1= first molar, M2= second molar, M3= third molar.

Figure 4. ln ((1 + Asx D/L)/(1 - Asx D/L)) vs estimated tooth age by tooth type for the Grantham teeth analysed. Ages for the Grantham population are those determined from dental development and dental wear. Y error bars represent 1st standard deviation about the mean for duplicate measurements from the same tooth. X error bars represent the errors in the morphological age estimates. m1= deciduous first molar, m2= deciduous second molar, P2=second premolar, M1= first molar, M2= second molar, M3= third molar.

Figure 5. ln ((1 + Asx D/L)/(1 - Asx D/L)) vs actual tooth age by tooth type for the Spitalfriedhof St Johann teeth analysed. Error bars represent 1st standard deviation about the mean for duplicate measurements from the same tooth. C=canine, P1= first premolar, P2=second premolar, M1= first molar, M2= second molar, M3= third molar.

Figure 6. Relationship between repeated age estimates from different teeth from the same individual (R^2=0.722). Modern (◆), Newcastle Blackgate (■), Grantham (△) and Spitalfriedhof St Johann (○). Line shown is x=y.

Table 1. Repeated age estimates produced from different teeth from the same individual (tooth types arranged in order of age at formation, with a second column where more than one of that tooth type was present), rounded to the nearest year. Confidence intervals of ± 9 years (modern), ± 8 years (Grantham), ± 12 years (Blackgate) and ± 20 years (Basel) apply [13, 14].

Individual	dm1	dm2	M1	I1	I1 (2)	I2	C	P1	P2	M2	M2 (2)	M3	M3 (2)
Modern 1				43	43	48							
Modern 2							42	44					
Modern 3							50	41					
Blackgate 1			18					8		9			
Blackgate 2			39							20			
Blackgate 3							37			36			
Blackgate 4	5	8											
Blackgate 5				31		29		23		36			
Blackgate 6								30	37				
Blackgate 7			28							20	18	35	
Grantham 1	1	5											
Grantham 2			38									29	
Grantham 3	1	2											
Grantham 4									39			33	
Basel 1			26									23	
Basel 2									30	40			
Basel 3			41						28				
Basel 4			62							48			
Basel 5								28	26				
Basel 6												37	26